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(21) International Application Number: PCT/US89/02820 (22) International Filing Date: 30 June 1989 (30.06.89) (30) Priority data: 214,959 5 July 1988 (05.07.88) US (71) Applicant: UNIVERSITY OF MARYLAND [US/US]; College Park Campus, College Park, MD 20742 (US). (72) Inventor: HANSEN, Norman, J. ; 9704 Cottrell Terrace, Silver Spring, MD 20903 (US). (74) Agents: KELBER, Steven, B. et al.; Oblon, Spivak, McClelland, Maier & Neustadt, Fourth Floor, 1755 Jef- ferson Davis Highway, Arlington, VA 22202 (US).		(81) Designated States: AT (European patent), AU, BE (Euro- pean patent), CH (European patent), DE (European pa- tent), DK, FR (European patent), GB (European pa- tent), IT (European patent), JP, KR, LU (European pa- tent), NL (European patent), NO, SE, SE (European pa- tent). Published <i>With international search report.</i>
(54) Title: LEADER SEQUENCE INDUCING A POST-TRANSLATIONAL MODIFICATION OF POLYPEPTIDES IN BACTERIA, AND GENE THEREFOR (57) Abstract The method by which polypeptides having resi- dues other than the 20 common amino acids are made is established. A leader peptide sequence, and its gene, are identified which induce or assist post- translational modifications of Cys, Thr and Ser in prokaryotes. The leader sequence may be used to in- duce the presence of covalent bonding sites in poly- peptides and can be expressed by either naturally oc- curring or artificial means. <pre> 12 24 36 48 60 AGTTGACGAATATTTAATAATTTTATTAATATCTTGATTTCTAGTTCCTGAATAATATA 72 84 96 108 120 GAGATAGGTTTATTGAGTCTTAGACATACTTGAATGACCTAGTCTTATAACTATACTGAC 132 144 156 168 180 AATAGAAACATTAACAAATCTAAAACAGTCTTAATCTATCTTGAGAAAGTATTGGTAAT 192 204 216 228 240 AATATTATTGTCGATAACGCGAGCATAATAACGGCTCTGATTAATTTCTGAAGTTTGT 252 ^ <--5' end of nisin mRNA 288 *** AGATACAATGATTTGCTTGAAGGAACACAAAATAAATTATAAGGAGGCACTCAAATG r.b.s. MET ***** AGTACAAAAGATTTTAACTTGGATTTGGTATCTGTTTCGAAGAAAGATTCAGGTGCATCA SerThrLysAspPheAsnLeuAspLeuValSerValSerLysLysAspSerGlyAlaSer *****--C---TC---C--T-TG--C-----G--C--C-----C-----C CCACGCATTACAAGTATTTGCTATGTACACCCGGTTGTAAACAGGAGCTCTGATGGGT ProArgIleThrSerIleSerLeuCysThrProGlyCysLysThrGlyAlaLeuMETGly 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 20-mer --C--T-----T--A--C-----CTC---C--T--GTCT--- TGTAACATGAAAACAGCAACTTGTGATTGTAGTATTCACGTAAGCAAATAACCAATCAA CysAsnMETLysThrAlaThrCysHisCysSerIleHisValSerLysTER 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 492 3' end of nisin mRNA--> AGGATAGTATTTTGTAGTTCAGACATGGATACTATCC </pre>		

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Description

Leader Sequence Inducing a Post-translational Modification of Polypeptides in Bacteria, and Gene Therefor

5 Technical Field

This invention pertains to the expression of proteins which require post-translational modification of their amino acid sequence before a mature form is reached. Such proteins exhibit amino acids other than
10 the 20 common amino acids coded for by the conventional nucleic acids. Specifically, a leader peptide sequence is identified which can induce post-translational modification of specific amino acids when expressed in conjunction with the precursor polypeptide. Methods of
15 forming improved compositions using this leader sequence are also addressed.

Background of the Prior Art

Polypeptides, including those having natural antibiotic activities, have been identified which
20 comprise amino acids other than the 20 common acids specified by the genetic code, as the expression products of bacteria, and other organisms. The structure of two of the more important ones, nisin and subtilin are set forth in FIGURE 1 of this application.

25 The presence in these polypeptides, and others, of the unusual amino acids lanthionine, β -methyllanthionine, D-alanine, dehydroalanine, and dehydrobutyrine clearly suggests that something other than ordinary protein biosynthesis directed by the genetic code is
30 involved in the expression of the mature forms of these

naturally occurring polypeptides. Nonetheless, research has demonstrated that the appearance of these polypeptides can be blocked by protein biosynthesis inhibitors. Hurst et al., Canadian Journal of Microbiology, 17, 1379-1384 (1971). It is also known that precursor peptides of the mature forms can be detected with antibodies against the mature peptide. Nishio et al., Biochemistry Biophysics Research Community, 116, 751-751 (1983). These observations, with other observations concerning nisin, subtilin and related proteins suggest a mechanism that involves primary biosynthesis of a precursor via a ribosomal mechanism, followed by post-translational modifications.

15 The activity of these proteins, and potential mutant variations thereof, are of sufficient commercial interest so as to generate substantial activity in the field of derived microorganisms containing foreign DNA fragments and coding for the protein's production.

20 U.S. Patent 4,716,115, issued to Gonzalez et al. is directed to just such a derived microorganism. However, the impossibility of obtaining a genetic sequence that codes directly for the mature protein, and the lack of information concerning the nature of the post-translational modification necessary to arrive at the mature protein, has prohibited the cloning of microorganisms containing the specific gene which encodes for these proteins, and perhaps more importantly, has frustrated attempts to produce random variants and site-specific mutated proteins, which quite probably can be arrived at having higher degrees of activity, or other enhanced properties.

30

Thus, it remains an object of the biotechnology field to arrive at a comprehensive understanding of the

mechanism by which the mature forms of these unusual amino acid-containing polypeptides are made, and to develop an expression vehicle for incorporating a gene which will specifically encode for the production of these peptides and which is suitable for the transformation of commonly available bacteria.

Disclosure of the Invention

The Applicants have identified gene leader sequences, which, when coupled with the gene encoding the precursor of a polypeptide, induces or participates in the post-translational modification of the precursor to obtain the mature form. The structure of the full gene, including probable ribosomal binding sites, confirms the post-translational modification model for the manufacture of these peptides.

The gene for the expression of the precursor, and ultimately, the mature protein, of subtilin appears in FIGURE 2. The leader sequence, which can be used to promote post-translational modification of other proteins which contain unusual amino acids, such as nisin and the like, is set forth specifically in FIGURE 3. A separate leader sequence, bearing significant homology with that for subtilin, is also identified, and the overall gene sequence is given in FIGURE 3.

Brief Description of the Drawings

FIGURE 1 is the conformational structure for the small antibiotic proteins nisin and subtilin, as determined by Gross et al., Protein Cross-linking, pages 131-153 (1977).

FIGURE 2 is the genetic base pair sequence for the entire digested fragment containing the gene which

encodes for the subtilin precursor peptide, including the leader fragment responsible for inducing post-translational modification. A putative ribosomal binding site is labeled R.B.S., the leader fragment has
5 astericks above it, and those amino acids of the precursor which undergo modification are set forth in bold face.

FIGURE 3 is an illustration giving the sequence for the gene coding for nisin, and the precursor
10 polypeptide corresponding thereto bearing the same types of markings and having the same meanings as FIGURE 2.

Best Mode For Carrying Out The Invention

To arrive at the gene for the polypeptide
15 precursor for the proteins of interest, and therefore, for the ultimate expression of the mature form of the protein, it is necessary to develop a gene probe, based on the putative amino acid precursor sequence of the protein in question. For ease of discussion, the
20 description herein will be first in the context of the gene and precursor for subtilin, although the same methodology has been employed to determine the full gene for the precursor of nisin, as is discussed subsequently and is applicable to additional genes
25 encoding proteins containing similarly unusual amino acids in the mature form as well.

SUBTILIN

Organism and culture conditions. Bacillus subtilis ATCC 6633, a subtilin-producing strain, was
30 obtained from the American Type Culture Collection, Rockville, MD. It was cultured in the high-sucrose Medium A of Nishio et al (1983), originally described

by Feeney et al (1948). It contains (per l) 100 g sucrose, 11.7 g citric acid, 4 g Na_2SO_4 , 4.2 g $(\text{NH}_4)_2\text{HPO}_4$, 5 g yeast extract (Difco), 100 ml of a salt mixture (7.62 g KCl, 4.18 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.543 g $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$), 0.49 g $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, and 0.208 g ZnCl_2 in 1000 ml of H_2O), and sufficient NH_4OH to bring the pH to 6.8-6.9 per liter. Stocks were maintained on LB plates (10 g tryptone, 5 g yeast extract, 10 g NaCl per l) containing 1.5% agar.

10 Clone isolation and hybridization procedures. A subtilin gene probe was designed based on the putative amino acid precursor sequence of subtilin. The mature subtilin molecule contains only 32 amino acids, and does not contain any regions of low codon degeneracy.

15 Therefore, instead of preparing a probe mixture which contained all possible sequences encoding a short stretch of amino acids in the subtilin precursor, a single long probe was synthesized according to the strategy of Lathe, Journal of Molecular Biology, 183, pp. 1-12 (1985). Ambiguous positions within codons

20 were chosen by educated guess, according to a codon frequency usage table constructed from the known B. subtilis gene which codes for alpha-amylase Yang et al, Nucleic Acids Research, 11, pp. 237-249 (1983).

25 Because one cannot predict the sequence homology between the probe and the target gene sequence, hybridization and wash conditions must be optimized empirically. The 96-mer "guessmer" was end-labeled

30 using polynucleotide kinase, purified on disulfide cross-linked BAC gels as described by Hansen et al (1982), and hybridized to EcoRI digests of total ATCC 6633 genomic DNA at 7°C temperature intervals in the range of 37-60°C, using a 6x Standard Saline Citrate

35 (SSC) salt strength. Separate strips were then washed,

using temperature increments of 4°C, in 2x SSC. The hybridization and wash conditions that gave the best combination of signal strength and specificity were chosen for subsequent screening of a partial Mbol library of ATCC 6633 DNA constructed in lambda J1. Hybridizations in which probe and target were highly homologous were carried out in the same hybridization buffer as above, but the hybridization temperature was 70°C, washes were done in 0.1x SSC at 52°C. DNA sequence analysis was done using the modified T7 polymerase "Sequenase" system supplied by United States Biochemical Corp.

RNA isolation and S1-mapping. Total RNA was isolated using the method of Ulmanen et al (1985). S1-mapping was performed by the method of Davis et al (1986), in which a synthetic oligonucleotide is used to prime second strand synthesis using single-strand M13 DNA which contains the cloned gene as template. Label was incorporated as ³²P from [alpha-³²P-dATP]. After a short labeling time, an excess of unlabeled dATP was added, and second strand synthesis was continued toward completion. An appropriate restriction enzyme was used to cut the double-stranded product, and the labeled strand was obtained by electrophoresis on a denaturing agarose gel, followed by autoradiography to locate the fragment, excision of the gel, and electroelution of the DNA. After electroelution, the DNA was extracted with 1:1 chloroform:phenol and precipitated with ethanol. The labeled fragment was hybridized to total mRNA at several different temperatures, and unhybridized single-strand nucleic acid was degraded using nuclease-S1. The product was electrophoresed on a denaturing sequencing gel alongside a set of dideoxy sequencing reactions generated using the same synthetic

oligonucleotide as primer. The location of the protected labeled DNA fragment with respect to the sequencing lanes identified the end of the mRNA.

RNA and protein analysis. Northern analysis was
5 done by electroblotting acrylamide gels of RNA
preparations onto Zeta-probe nylon membrane (Bio-
Rad). Proteins were analyzed by electrophoresis on the
polyacrylamide gel system of Swank and Munkres (1971),
and silver-stained using Bio-Rad reagents. Subtilin
10 activity was measured as for nisin, described by Morris
et al (1984).

Using the above materials and methods, fragments
which contained the sequence hybridizing with the
guessmer were cloned into M13 and sequenced. The
15 sequence was searched for homology to the subtilis gene
probe, and also computer-translated in all reading
frames. These were searched for the putative subtilin
precursor sequence. A perfect match was found, which
contains the exact sequence of 32 residues. The
20 sequence is set forth in FIGURE 3.

As noted, this sequence includes a portion
encoding a precursor polypeptide, which contains
serines, threonines and cysteines which undergo
modification after translation, to arrive at the mature
25 protein, having the unusual amino acids noted. The
(-10) region corresponds closely to a consensus
prokaryotic promoter (TATAAT) as observed in other
bacteria, Siebenlist et al., Cell, 20, pages 269-281
(1980). The putative ribosome binding site is labeled
30 as RBS and encompasses a 12 base pair sequence that is
typical of those observed in B. subtilis, as reported
by Band et al., DNA, 3, pages 17-21 (1984). It should
be noted that it is positioned so that translation
initiation would begin at the immediate downstream Met

codon, which initiates the leader sequence of this invention. It should be noted that the subtilin precursor peptide leader region, which plays a role in the transport of subtilin outside the cell, is unusual in comparison to sequences of other prokaryotic exported proteins.

NISIN

The above approach has been duplicated for the antibiotic nisin, and the resulting gene sequence, coding for the precursor, is set forth in FIGURE 3 attached hereto.

Bacterial strains, cloning vectors, and culture conditions. Nisin-producing Streptococcus lactis ATCC 11454 was obtained from the American Type Culture Collection (Rockville, MD). Strains were stored at -20°C in ATCC Medium 17 (100 g skim milk powder, 100 g tomato juice, 5 g yeast extract to pH 7.0) containing 25% glycerol. Working stocks were maintained on 1.2% LB agar plates (10 g Bacto-tryptone, 5 g Bacto-yeast extract, 10 g NaCl per liter). M17 culture medium (8), consisting of 5 g Bacto-peptone (Difco), 5 g Bacto-soytone (Difco), 2.5 g yeast extract (Difco), 5 g beef extract (Difco), 0.5 g ascorbic acid, 5 g lactose (or glucose) 19 g beta-disodium glycerophosphate (Eastman), and 0.12 g anhydrous $MgSO_4$ per liter, was used to culture S. lactis for nisin production, genomic library construction, and total RNA isolation. The organism was grown at 32°C without aeration using a 2% inoculum into an appropriate volume of M17 medium.

Bacillus cereus T spores used in the assay for nisin production were prepared and stored as described in the art. Antibiotic activity assays were performed as previously described using fractions of the S.

lactis culture supernatant.

DNA isolation procedure. S. lactis ATCC 11454 was incubated in 500 ml of M17 medium for 30 hours at 32°C without aeration. Cells were collected by

5 centrifugation, and washed in 25 ml PBS (8 g NaCl, 1.4 g Na₂HPO₄, 1.2 ml 1 N HCl per liter). The cells were resuspended in 15 ml 50 mM Tris-HCl (pH 7.6) and subsequently digested with 33 micrograms per ml

10 mutanolysin (Sigma) for 15 minutes at 37°C with gentle agitation (12). Then 5 ml of STEP solution (13) (0.5% SDS, 50 mM Tris-HCl in 0.4 M EDTA, and 1 mg per ml proteinase K) was added and incubation performed at 37°C for 30 min with occasional mixing. The mixture was extracted with 1 volume of CHCl₃, 1 volume 50:50

15 phenol:CHCl₃, and finally with 1 volume CHCl₃. One-tenth volume 3 M Na acetate and 2 volumes ethanol were added; the DNA was spooled, and resuspended in 20 ml 50 mM Tris-HCl and 4 mM EDTA containing 50 micrograms per ml of pancreatic RNase (Sigma). The solution was

20 dialyzed against a buffer of 50mM Tris-HCl and 4 mM EDTA for 16 hours at 4°C with one buffer change. The DNA was ethanol-precipitated two times in the presence of 2.5 M ammonium acetate and finally dissolved in 2 ml 10 mM Tris-HCl, pH 7.6.

25 Probe construction, radiolabeling, and hybridization procedures. Several different probes were used to search for the nisin gene in S. lactis ATCC 11454 DNA. Hybridization conditions were optimized as previously described (2). Two oligomeric

30 probes were prepared by chemical synthesis using a Biosearch Model 8700 DNA synthesizer. One was a 20-mer mixed probe designed against a region of low codon degeneracy within the putative nisin precursor sequence. The second was a single sequence 103-mer

oligonuenceotide probe designed using the strategy of Lathe. A natural DNA probe was also employed, which was a 1.1 kb restriction fragment containing the subtilin gene that had previously been cloned from
5 Bacillus subtilis ATCC 6633 (2).

Library construction and isolation of the nisin gene. A total genomic library of S. lactis ATCC 11454 DNA in lambda J1 was constructed and screened as described above. Positive clones were mapped by
10 restriction analysis and subcloned into pUC9 and pTZ19U plasmid vectors for further analysis, and into M13mpl8 and M13mpl8 for sequencing. Sequence determination was performed by the dideoxy termination method using modified T7 polymerase and the protocol in a Sequenase
15 kit obtained from the United States Biochemical Company.

RNA isolation and Northern blot analysis. Total RNA isolation was performed according to the method of Ulmanen et al. RNA fractionation was performed on a
20 denaturing acrylamide gel, electroblotted onto Zeta-probe (Biorad) nylon membrane, and hybridized as described above.

Protein analysis. Proteins were analyzed by electrophoresis on the polyacrylamide gel system of
25 Swank and Munkres, and silver-stained using Bio-Rad reagents. Nisin activity was determined by the method of Morris et al.

Discussion

Thus, the mode by which subtilin, nisin, and other
30 proteins containing unusual amino acids not encoded by the genetic code is established. Specific leader sequences encoded within the genes for subtilin and nisin shown in FIGURES 2 and 3 required for post-

translational modification of specific amino acids, including precursor residues Ser, Thr and Cys, which are converted to the unusual amino acids referred above, undergoing reactions which include dehydration, and potential electrophilic addition reactions involving stereoinversion to generate thioether cross-linkages and D-amino acids. Genes coding for the precursor polypeptide, including the leader, can be inserted through conventional technologies into any expression vehicle, which, e.g., for nisin, include Streptococcus lactis as a natural producer, and the expression bacteria set forth, e.g., in U.S. Patent 4,716,115. Similar expression vehicles can be identified for other proteins.

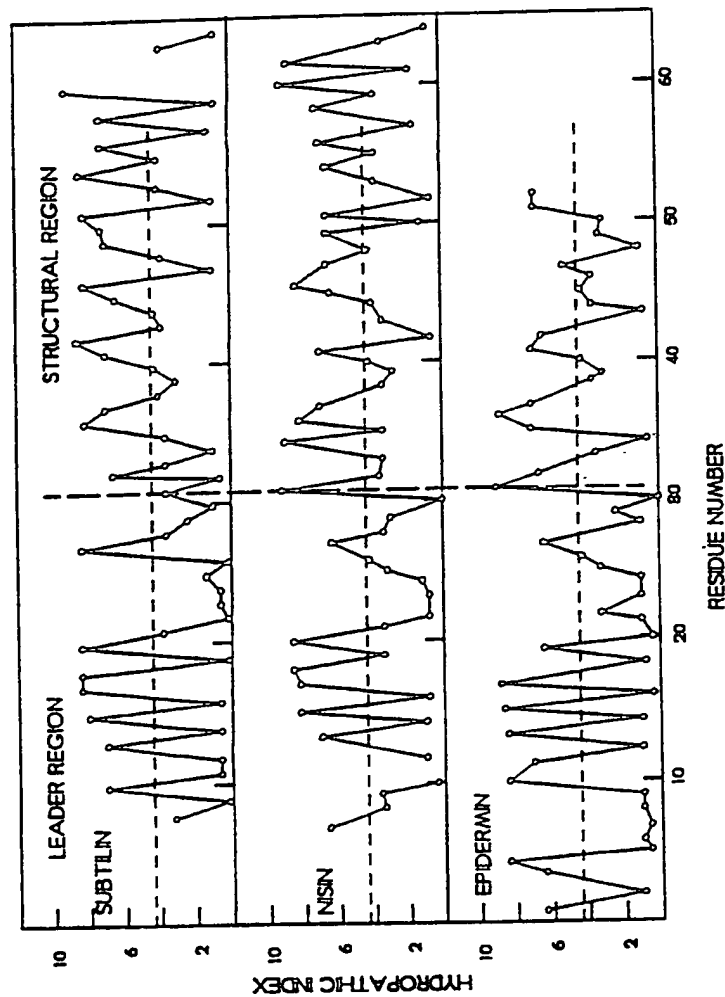
Subsequent to the invention addressed herein, the gene sequence for epidermin, another lanthionine-containing polypeptide antibiotic was published by Schnell et al, Nature, 333, pp. 276-278 (1988). Although the amino acid residues of the leader sequences for the three antibiotics reflect sufficient homology to indicate a common evolutionary origin, it is clear that at this time, there are significant differences in the amino acid sequences of each, and their corresponding gene sequences. However, as reflected in Table 1, the hydropathic index of the three leader amino acid sequences are astonishingly similar. Specifically, adjacent to the structural regions, there is a region of high hydrophilicity, followed by a region more distal from the structural region, which, on average, is neutral, but tends to alternate between a hydrophilic and a hydrophobic residue. Indeed, placed on the same graph, there is an amazing correlation with regard to these residues. This correlation continues down to the fact that each

leader region reflects an interruption in the hydrophilic residues with one hydrophobic residue, at the exact same location in each case. Thus, the invention herein embraces not only the recognition that modification is accomplished by encoding a leader region which directs or aids in achieving modification in the structural region, but extends to the recognition that the leader region can be generally characterized as having a portion proximal to the structural region which is hydrophilic in nature, complemented by a more distal portion wherein hydrophilic and hydrophobic residues alternate to give an overall neutral value. Emperically, the three examples set forth herein all include the presence of a single hydrophobic residue in the hydrophilic portion adjacent the structural region. As of the filing date of this application, it is unknown whether the presence of such a residue is essential for achieving the post-translational modifications necessary. However, given the state of skill in the art, routine experimentation can determine the necessity of such a presence, together with various alternatives, which may improve modification efficiency.

The available technology also allows the manufacture of a gene encoding a mature protein, from the gene for the structural region only, which in many cases can be determined in a relatively straightforward manner, i.e., prediction based on the amino acid sequence followed by hybridization and sequence analysis. The effect of the leader sequence of this invention on specific amino acids also provides a novel means for achieving site-specific mutagenesis without resort to DNA modification. Thus, for example, it has been reported that deletion or replacement of various

residues, such as cysteine, may improve biological activity. See, e.g., U.S. Patent 4,518,584. Additionally, novel mutants of naturally-occurring peptides are quite likely to possibly exhibit higher activities, or better specificities for certain biological functions. These can now be prepared by insertion of the genetic code for the leader sequence of this invention in front of the gene encoding the expression of a naturally-occurring polypeptide, which will then undergo the post-translational modification directed by the leader sequence, eliminating or modifying the residues in question.

TABLE 1



It should also be noted, of course, that where it is desired to secure substantial expression of the precursor, and not the peptide itself, this can now also be achieved, by specific excision of the leader
5 fragment from the gene encoding the peptide precursor. In the absence of the leader sequence of this invention, it is the precursor which will be expressed, without direction to undergo post-translational modification.

10 Another feature of the invention of this application is the capability of designing "targeted proteins", or proteins which, by virtue of the presence of the unusual amino acids dehydroalanine and dehydrobutyrine, can be covalently attached to a
15 "target". Thus, using structural variants, which could recognize and select for specific targets, the leader fragment can be employed to induce "binding sites", to develop a covalent bonding "antibody", to neutralize specific toxins, to select out specific material,
20 etc. All these modifications are well within the skill of the ordinary practitioner and the expanding biotechnology arts, and so represent immediate applications of the discovery of the leader sequence disclosed herein.

25 Applications of this invention are not limited to the modification of existing proteins. Given current abilities to synthesize DNA sequences, specific polypeptides can be encoded by artificial clones and targeted for specific uses. As an example, given the
30 crosslinking ability of the unusual amino acids produced through this invention, an adhesive can be prepared specific for a given substrate, e.g., carbon fibres, which due to the capability of the unusual amino acids generated by modification to form covalent

linkages, can firmly bond to the substrate. The availability of amino acids allows the designer to introduce as an adhesive any desired amount of hydrophobicity, hydrophilicity, etc., to overcome
5 problems encountered in currently used adhesives, such as epoxies.

Of course, specific applications will generate mutations of the leader sequence of this invention, and other specific variants. So long as these variants
10 retain the essential biological function of inducing or assisting in post-translational modification, they remain within the scope of this invention.

It should be noted that a publication detailing the identification of the leader sequence by the
15 Applicant, in conjunction with Sharmila Banerjee will appear in the Journal of Biological Chemistry, Vol. 263, proposed publication date July 5, 1988.

The exact mechanism by which post-translational modification is induced is unclear. Without being
20 bound to any theory, it is noted that the subtilin precursor exhibits residues in the leader sequence that initially alternate between high hydrophilic and high hydrophobic nature, becoming highly hydrophilic near the structural region, which, in contrast is strongly
25 hydrophobic. This should be contrasted with usual leader regions for exported proteins of prokaryotes, which generally have a quite hydrophobic region, and contain basic residues, not the acidic residues of the invention. This suggests the post-translational
30 modifications occur at a compartmentalized site, which the unusual leader sequence assists in targeting or directing the precursor too. It is expected that other proteins will participate in the modification mechanisms. Enzymes necessary to effect the essential

chemical reactions localized at or near the cell membrane.

This invention has been described in specific detail with regard to specific proteins, materials and
5 methods. Except where necessary for operability, no limitation to these specific materials is intended, nor should such a limitation be apprehended, outside the express limitations of the claims appended hereto. In particular, use of the leader sequence of this
10 invention in conjunction with virtually any prokaryotic expression vehicle, specifically bacteria, is contemplated.

Claims

1. A gene leader fragment encoding a peptide leader sequence which induces post-translational modification of amino acids selected from the group consisting of Cys, Ser, Thr and mixtures thereof, said
5 fragment having the sequence

ATG TCA AAG TTC GAT GAT TTC GAT TTG GAT GTT GTG AAA GTC
TCT AAA CAA GAC TCA AAA ATC ACT CCG CAA.

2. A polypeptide sequence which, when attached as
10 a leader to a protein precursor which undergoes post-translational modification, assists in inducing said modification, comprising a polypeptide having the biological function of the amino acid sequence
Met Ser Lys Phe Asp Asp Phe Asp Leu Asp Val Val Lys Val
15 Ser Lys Gln Asp Ser Lys Ile Thr Pro Gln.

3. A genetic sequence encoding a polypeptide precursor of subtilin in bacteria, having the sequence
ATG TCA AAG TTC GAT GAT TTC GAT TTG GAT GTT GTG AAA GTC
TCT AAA CAA GAC TCA AAA ATC ACT CCG CAA TGG AAA AGT GAA
20 TCA CTT TGT ACA CCA GGA TGT GTA ACT GGT GCA TTG CAA ACT
TGC TTC CTT CAA ACA CTA ACT TGT AAC TGC AAA ATC TCT AAA

4. A preparation of a precursor polypeptide which, when expressed in bacteria, is converted after translation to the protein subtilin.

- 25 5. The polypeptide of Claim 4, wherein said polypeptide has the residue sequence
Met Ser Lys Phe Asp Asp Phe Asp Leu Asp Val Val Lys Val
Ser Lys Gln Asp Ser Lys Ile Thr Pro Gln Trp Lys Ser Glu
Ser Leu Cys Thr Pro Gly Cys Val Thr Gly Ala Leu Gln Thr
30 Cys Phe Leu Gln Thr Leu Thr Cys Asn Cys Lys Ile Ser Lys

6. A gene leader fragment encoding a peptide leader sequence which induces post-translational modification of amino acids selected from the group

consisting of Cys, Ser, Thr and mixtures thereof, said fragment having the sequence
ATG AGT ACA AAA GAT TTT AAC TTG GAT TTG GTA TCT GTT TCG
AAG AAA GAT TCA GGT GCA TCA CCA CGC

- 5 7. A polypeptide sequence which, when attached as a leader to a protein precursor which undergoes post-translational modification, assists in inducing said modification, comprising a polypeptide having the biological function of the amino acid sequence
10 MET Ser Thr Lys Asp Phe Asn Leu Asp Leu Val Ser Val Ser Lys Lys Asp Ser Gly Ala Ser Pro Arg

8. A preparation of a precursor polypeptide which, when expressed in bacteria, is converted after translation to the protein nisin.

- 15 9. A method for inducing site-specific mutagenesis in a desired polypeptide having, as translated at least one amino acid selected from the group consisting of Cys, Thr and Ser comprising:

preparing a DNA fragment encoding a precursor
20 polypeptide comprising said desired polypeptide and a leader fragment attached thereto, said leader fragment comprising a region of residues adjacent to said desired polypeptide which are predominally hydrophilic, and a more distal region having an overall neutral
25 hydropathic index but wherein substantially all adjacent residues have opposite hydropathic indices, and

inserting said fragment in the DNA of an expression vehicle to express and modify the precursor
30 polypeptide.

10. The method of Claim 9, wherein said expression vehicle is a prokaryote.

11. The method of Claim 10, wherein said expression vehicle is a bacteria.

12. A polypeptide expressed by an expression vehicle comprising at least one residue not encoded by DNA, said residue being at a predetermined site, caused by post-translational modifications of a precursor polypeptide comprising a leader fragment having a region of highly hydrophilic residues adjacent a structural region, said leader fragment having a second region of residues of substantially alternatively hydropathic index, said structural region bearing a Cys, Ser or Thr residue at this site corresponding to said predetermined site, said polypeptide being one not encoded by said expression vehicles naturally occurring DNA genome.

13. A process for the expression of site directed mutants of nisin and subtilin or similar peptides where the modification is in the mature sequence and results in substantially altered biological properties of the mature peptide.

14. A process for the expression of nisin and subtilin by recombinant microorganisms produced by transformation with plasmids which incorporate the entire structural genes given herein.

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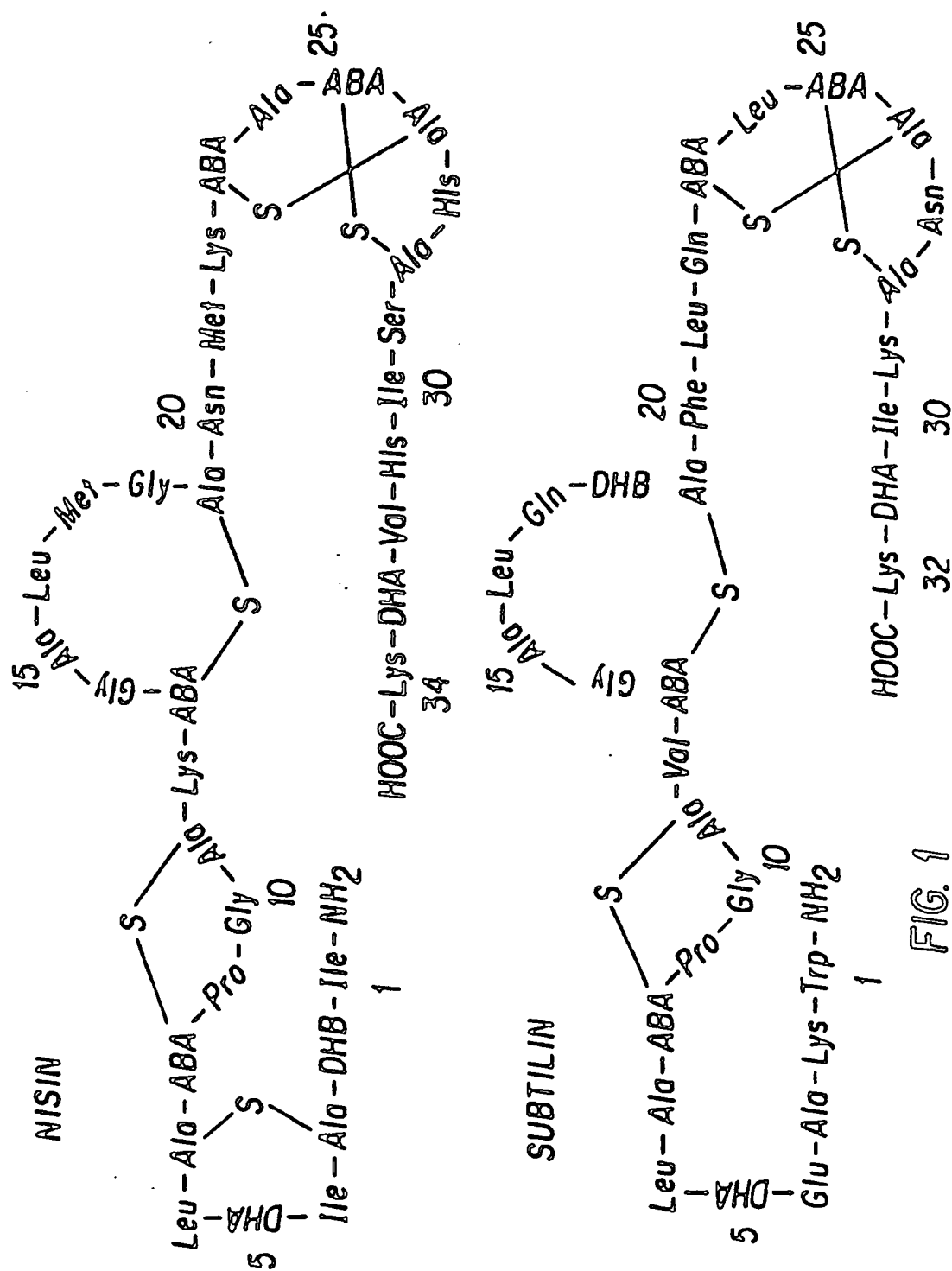


FIG. 1

SUBSTITUTE SHEET

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12      24      36      a gat ca      60
CCG GAC AGG AGT ATT TTA AGG AAG AGC TTC AAG AGT TAA ACA AAA GAT CAT GAG CTA CTA

          g att cca      (mRNA)
TGA CAA GGA TTA TAT CTT TGG ATT CCA TAA CTA TGA ATC AAT GGA AGG GGA CGA AGC AGT

          (-10)      (+1)
          144
ACC TTT GCA GTA CGT TGG TTT GTT GGA TGG AGC TGT AGG TGT AGG CTT AGG GGT ATT AAA

          192      204      216      228
CAT GGA ATT AGG CTC AAA AAC AGA TTG GAC AAA AGC ATT ATT AAT TTA ATA AAA AAA GGA

          252      264      276
AAA AAA TGA TAA AAT CTT GAT ATT TGT CTG TTA CTA TTT AGG TAT TGA AAG GAG GTG ACC

          (r.b.s.)
          SSS SSS S

*****
AAT ATG TCA AAG TTC GAT GAT TTC GAT TTG GAT GTT GTG AAA GTC TCT AAA CAA GAC TCA
MET Ser Lys Phe Asp Asp Phe Asp Leu Asp Val Val Lys Val Ser Lys Gln Asp Ser
          (leader region)

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FIG. 2

SUBSTITUTE SHEET

4/4

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      12      24      36      48      60
AGTTGACGAATATTTAATAATTTTATTAATATCTTGATTTTCTAGTTCCTGAATAATATA

      72      84      96     108     120
GAGATAGGTTTATTGAGTCTTAGACATACTTGAATGACCTAGTCTTATAACTATACTGAC

      132     144     156     168     180
AATAGAAACATTAACAAATCTAAAACAGTCTTAATTCTATCTTGAGAAAGTATTGGTAAT

      192     204     216     228     240
AATATTATTGTCGATAACGCGAGCATAATAAACGGCTCTGATTAAATTCTGAAGTTTGT
      252 ^ <--5' end of nisin mRNA 288 ***
AGATACAATGATTTGTTCTGAAGGAAC TACAAAATAAATTATAAGGAGGCACTCAAAATG
                                   r.b.s.      MET

```

```

AGTACAAAAGATTTTAACTTGGATTTGGTATCTGTTTCGAAGAAAGATTCAGGTGCATCA
SerThrLysAspPheAsnLeuAspLeuValSerValSerLysLysAspSerGlyAlaSer

```

```

*****--C---TC---C--T-TG--C-----G--C--C-----C-----C
CCACGCATTACAAGTATTTTCGCTATGTACACCCGGTTGTAAACAGGAGCTCTGATGGGT
ProArgIleThrSerIleSerLeuCysThrProGlyCysLysThrGlyAlaLeuMETGly
  1  2  3  4  5  6  7  8  9 10 11 12 13 14 15 16 17 18

```

```

      20-mer
--C--T-----T--A--C-----CTC---C--T--GTCT---      480
TGTAACATGAAAACAGCAACTTGTCAATTGTAGTATTACGTAAGCAAATAACCAAATCAA
CysAsnMETLysThrAlaThrCysHisCysSerIleHisValSerLysTER
 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34

```

```

      492 3' end of nisin mRNA-->|
AGGATAGTATTTTGTAGTTCAGACATGGATACTATCC

```

FIG.3

N⁺57N

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No. **PCT/US89/02820**

I. CLASSIFICATION OF SUBJECT MATTER

According to International Patent Classification (IPC) or to both National Classification and IPC
IPC(4): CO7H 21/04; CO7K 7/10; CO7K 15/00; C12P 21/00

II. FIELDS SEARCHED

Minimum Documentation Searched ⁷

Classification System

Classification Symbols

U.S. 435/68,70,71,91,172.1,172.3,252.3,252.31-252.35,320;
 536/27;530/300,325,326,350;935/11,47,49,72

Documentation Searched other than Minimum Documentation
 to the Extent that such Documents are Included in the Fields Searched ⁸

CA File 1967-1989 IG Suite Sequence Files:
 BIOSIS File 1967-1989 GenBank, EMBL, PIR

III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹

Category [*]	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
Y	US, A, 4,716,115 (GONZALEZ ET AL) 29 December 1987. See entire document.	6-8
X,P Y,P	G. W. BUCHMAN ET AL, "Structure, expression, and evolution of a gene encoding the precursor of nisin, a small protein antibiotic", Journal of Biological Chemistry, Volume 263, number 31, pages 16260-16266, published November 1988 by The American Society for Biochemistry and Molecular Biology, Inc. (Baltimore, MD, USA). See entire document.	6-12 1-5

^{*} Special categories of cited documents: ¹⁰

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"A" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

Date of Mailing of this International Search Report

08 August 1989

28 SEP 1989

International Searching Authority

Signature of Authorized Officer

ISA/US

James Martinell

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _____, because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claim numbers 13 and 14, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out ¹³, specifically:
 Claims 13 and 14 are process claims that fail to recite a process step. Claim 14 is an omnibus-type claim in that it refers to the entire application (See PCT Rule 6.2 (a)).

3. ☐ Claim numbers _____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

III DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
$\frac{X}{Y}$	A. HURST ET AL, "Observations on the conversion of an inactive precursor protein to the antibiotic nisin", Canadian Journal of Microbiology, Volume 17, pages 1379-1384, published 1971 by the National Research Council of Canada (Ottawa, Canada). See entire document.	$\frac{7,8}{6}$
$\frac{X}{Y}$	C. NISHIO ET AL, "Peptide antibiotic subtilin is synthesized via precursor proteins", Biochemical and Biophysical Research Communications, Volume 116, number 2, pages 751-758, published October 31, 1983 by Academic Press, Inc. (New York, NY, USA). See entire document..	$\frac{4,5}{2}$
$\frac{X}{Y}$	S. BANERJEE ET AL, "Structure and expression of a gene encoding the precursor of subtilin, a small protein antibiotic", Journal of Biological Chemistry, Volume 263, number 19, pages 9508-9514, published 28 June 1988 by the American Society for Biochemistry and Molecular Biology, Inc. (Baltimore, MD, USA). See entire document.	$\frac{1-5}{9-12}$